Activity of Bisnaphthalimidopropyl Derivatives against *Trypanosoma brucei*

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ABSTRACT

Current treatments for African trypanosomiasis are either toxic, costly, difficult to administer, or prone to elicit resistance. This study evaluated the activity of bisnaphthalimidopropyl (BNIP) derivatives against Trypanosoma brucei. BNIPDiaminobutane (BNIPDabut), the most active of these compounds, showed *in vitro* inhibition in the single-unit nanomolar range, similar to the activity in the reference drug pentamidine, and presented low toxicity and adequate metabolic stability. Additionally, using a murine model of acute infection and live imaging, a significant decrease in parasite load in BNIPDabut-treated mice was observed. However, cure was not achieved. BNIPDabut constitutes a new scaffold for antitrypanosomal drugs that deserves further consideration.

TEXT

African trypanosomiasis is an infectious disease caused by parasites of the species Trypanosoma brucei. The parasite is transmitted by an insect vector, the tsetse fly (Glossina spp.). The disease is distributed mainly on the African continent, with distinct subspecies causing different forms of human disease. T. brucei gambiense produces a chronic form of infection that may last for years and was responsible for nearly 98% of trypanosomiasis cases in the past decade. The acute form is caused by T. brucei rhodesiense and usually kills the host within weeks, accounting for the remaining 2% of reported cases (1, 2).

Since vaccination remains elusive and vector control strategies are frequently insufficient, chemotherapy is still the most efficient option for controlling the disease (2–5). However, the drugs in current use have many drawbacks, mostly related to cost, effectiveness, toxicity, difficulty of administration, and appearance of resistance (6). Therefore, the development of new drugs is urgently needed.

Bisnaphthalimidopropyl (BNIP) derivatives have been shown to possess anticancer activity (7–11) and to have activity against a related trypanosomatid, Leishmania infantum (12–14). The potential activity of three BNIP derivatives previously synthetized (>96% pure [10, 11, 15]), namely, BNIPDiaminobutane (BNIPDabut), BNIPDiaminoheptane (BNIPDahep), and BNIPDiaminooctane (BNIPDaoct) (Fig. 1A), against T. brucei brucei Lister 427 bloodstream forms (BSFs), was investigated. These derivatives were selected from a series of compounds on the basis of preliminary studies of bioavailability and *in vitro* and *in vivo* activity against T. brucei and L. infantum (12; our unpublished data). The *in vitro* antiparasitic activity was assessed using a resazurin assay, as previously described, with minor modifications (incubation with 10^3 parasites/well, in 200 µl [16]). All three BNIPs demonstrated a potent inhibitory effect on parasite growth, with a 50% inhibitory concentration (IC_{50}) within the nanomolar range (Fig. 1B; Table 1). BNIPDabut was the most active compound, with a mean IC_{50} (± standard deviation [SD]) of 2.4 ± 1.0 nM, similar to that of the reference drug pentamidine (mean IC_{50} [± SD], 2.9 ± 0.7 nM; Table 1). Since this class of compounds has been described as a group of inhibitors of the L. infantum Silent information regulator 2-related protein 1 (*Li*Sir2rp1; GenBank accession number AAN39039.1) (15), we evaluated whether inhibition of the T. brucei orthologue *Tb*Sir2rp1 (GenBank accession number AAX70528.1) is a possible mechanism of action. Whereas BNIPDabut was shown to inhibit the NAD^+-dependent deacetylase activity of *Tb*Sir2rp1 with an IC_{50} (± SD) of 155 ± 42 µM, suggesting that this is not the major mechanism of action (data not shown), *Li*Sir2rp1 was inhibited with an IC_{50} (± SD) of 35.0 ± 5.8 µM (15). The 47% identity between *Li*Sir2rp1 and *Tb*Sir2rp1 obtained by protein sequence alignment (Clone Manager 9, BLOSUM 62 scoring matrix)

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might explain the differences observed (17). Moreover, no correlation between the enzymatic inhibition and activity toward T. brucei parasites was observed. To evaluate *in vitro* toxicity toward mammalian cells, all the compounds were studied with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (18) in THP-1–derived macrophages and two primary cell cultures, rat cortical neurons and mouse hepatocytes (Table 2). The 50% cytotoxic concentration (CC_{50}) values for these molecules translate into selectivity indexes (SIs) higher than 100 (SI = CC_{50}/IC_{50}). All the BNIP derivatives exhibited a high SI, with BNIPDabut in particular being at least 800 times more selective toward T. brucei parasites. All the BNIPs had potency and selectivity that warranted additional characterization (Table 2). To further evaluate the potential toxic effects of BNIPs in host cells, a set of *in vitro* assays was performed in hepatocytes and neuronal primary cells. These assays evaluated different possible mechanisms of toxicity based on (i) reactive oxygen species determination (chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate [CM-H2DCFDA] probe, by high content analysis [HCA]), (ii) mitochondrial dysfunction (tetramethylrhodamine, methyl ester [TMRM] probe, by HCA), (iii) membrane integrity (lactate dehydrogenase quantification), (iv) apoptosis (caspase 3/7 activation), (v) either DNA damage for hepatocytes (H2AX antibody, by HCA) or neurite outgrowth for neurons (anti-tubulin III antibody, by HCA), (vi) cell viability as measured by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt probe], and (vii) Hoechst staining for nuclear detection. Nimesulide (400 μM) was included as a positive control and the vehicle as a neutral control (19–21). The relative percent deviation from the neutral control was quantified and assigned with a number from 0 to 5 (0 [0% to 20% deviation], 1 [20% to 40%], 2 [40% to 60%], 3 [60% to 100%], 4 [100% to 1,000%], 5 [>1,000% deviation]). The sum of these values was ranked posteriorly to create combined injury criteria that varied from no injury (0) to low injury (1 to <5), moderate injury (≥5 to <12), and high injury (≥12). All BNIPs showed a dose-dependent injury score close to that of pentamidine in both cell types (Fig. 2A and B). BNIPDabut had a toxicity profile indistinguishable from that of the reference drug pentamidine.

To infer metabolic stability, mouse microsomes were incubated over 45 min with each compound at 5 μM, and each drug was quantified by liquid chromatography-tandem mass spectrometry. Similarly to pentamidine, BNIPDabut was more stable than BNIPDahep and BNIPDaoct; 95% to 100% of the drug was not metabolized (Table 3). This high metabolic stability is an indicator that the molecule is not easily subjected to common inactivation or loss of potency by reactions catalyzed by liver enzymes and is kept intact in circulation for longer periods.

To determine the pharmacokinetics of BNIPDabut, a 10-mg/kg dose was administered to BALB/c mice by intravenous injection. Five minutes later, a concentration of 58 nM was achieved and, during the following 24 h, remained higher than 41 nM (data not shown); thus, the concentration was approximately eight times higher than the calculated IC_{90}.

Taking into consideration the previous results, we chose BNIPDabut for *in vivo* efficacy studies. All the experiments involving animals were carried out in accordance with the Institute for Molecular and Cell Biology Animal Ethics Committees and the Portuguese and European Authorities for Animal Health guidelines. T. brucei brucei Lister 427 parasites were transfected with a construct kindly provided by M. Taylor, in which the red-shifted luciferase gene (*PpyRE9h*) is flanked by 5′VSG/3′tubulin (22). After transfection, clones were screened for bioluminescent signal, and the ones that expressed the highest levels were selected. Their *in vitro* growth was compared with that of wild-type parasites and was found to be similar (data not shown). *In vitro* detection limits were also analyzed for BSFs in a 96-well plate and determined to be approximately 2,500 cells (data not shown). Female BALB/c mice were

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inoculated intraperitoneally with 10^4 BSFs. Three days after infection, five groups of mice (n = 4 per group) were treated intravenously with (i) saline, (ii) pentamidine at 2.5 mg/kg/day, (iii) dimethyl sulfoxide (DMSO) at 16.7%, or (iv) BNIPDabut at a dose of 10 or 20 mg/kg/day. Pentamidine was administered for 4 days, while BNIPDabut and the respective vehicle (DMSO at 16.7%) were administered for 6 days (Fig. 3A). No adverse effects were observable following any administration regimen. Treatment efficacy was followed through whole-animal live imaging using an IVIS Lumina LT (PerkinElmer). Parasitemia was also assessed, and animals were euthanized after reaching a parasite concentration of 10^8 parasites/ml. Similarly to pentamidine, two administrations of either 10 or 20 mg/kg BNIPDabut efficiently reduced parasitemia below the detection limit (5×10^4/ml) (Fig. 3B). However, whole-mouse imaging revealed that a reduction of bioluminescent signal to the background level (obtained with noninfected mice) was achieved only in mice treated with 20 mg/kg of BNIPDabut during at least 5 consecutive days, as opposed to 2 days of treatment for the pentamidine group. Although treatment with 20 mg/kg BNIPDabut apparently cleared the infection, cure was not achieved; the parasite burden relapsed when treatment was stopped (Fig. 3B). Indeed, BNIPDabut (10 and 20 mg/kg) treatment increased survival of the mice, but in contrast to those that received pentamidine, these animals were not cured (Fig. 3C). A hypothesis is that BNIPDabut, although highly trypanocidal, cannot reach and clear all parasites due to potency, distribution, or both. A frequent observation was that the recurrence of parasitemia was preceded by imaging of parasite loads in the peritoneal zone, where animals were originally injected with the parasites. It has been demonstrated that trypanosomes invade extravascular tissues as a defense mechanism against host immunity and that this process may be related to relapses after treatment interruption (23, 24). Indeed, the presence of parasites in the extravascular tissues might explain the discrepancy of radiance values between days 4 and 12, while average parasitemia levels remained similar in mice treated with BNIPDabut (10 or 20 mg/kg) (Fig. 3C). Nonetheless, it remains to be elucidated whether BNIPDabut is active in a mouse model of late-stage disease, as this is the central objective in drug discovery against human African trypanosomiasis. Additional chemical modifications to BNIPDabut may improve potency and/or distribution of the drug while maintaining or improving the toxicity and metabolism profiles.

In conclusion, this work demonstrates that BNIPDabut has potent *in vitro* and *in vivo* antitrypanosomal activity with acceptable toxicity and high metabolic stability. However, chemical modifications are needed to improve its pharmacodynamic and/or pharmacokinetic properties.

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TABLES & FIGURES

Table 1. In vitro activity of BNIP derivatives against *Trypanosoma brucei brucei* L427 bloodstream forms.

Compound	IC_{50} ± SD (nM)	IC_{90} ± SD (nM)
Pentamidine	2.94 ± 0.74	5.26 ± 0.58
BNIPDabut	2.35 ± 0.99	3.83 ± 1.40
BNIPDahep	14.32 ± 1.21	23.07 ± 1.01
BNIPDaoct	26.15 ± 10.43	63.36 ± 21.19

Table 2. In vitro cytotoxicity of BNIP derivatives in different cell types.

	CC_{50} ± SD (μM) in:			Selectivity index in[a]:		
Compound	THP-1[b]	Hepatocytes	Neurons	THP-1[b]	Hepatocytes	Neurons
Pentamidine	47.73 ± 3.32	18.21 ± 0.66	8.23 ± 0.88	16,259	6,203	2,803
BNIPDabut	5.90 ± 0.40	9.19 ± 0.06	2.06 ± 1.69	2,514	3,916	878
BNIPDahep	3.34 ± 0.11	4.23 ± 0.48	2.31 ± 1.65	233	295	161
BNIPDaoct	3.88 ± 0.59	18.35 ± 4.58	3.97 ± 1.30	148	702	152

[a] Selectivity index = CC_{50} cell line/IC_{50} *T. brucei*.
[b] THP-1 derived macrophages.

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Table 3. Mouse microsomal stability.

Compound	Metabolic stability (%)	*In vitro* intrinsic clearance (µl/min/mg protein)	Degradation non-NADPH dependent (%)
Pentamidine	95–100		<5
BNIPDabut	95–100		<5
BNIPDahep	85	7	<5
BNIPDaoct	64	20	9

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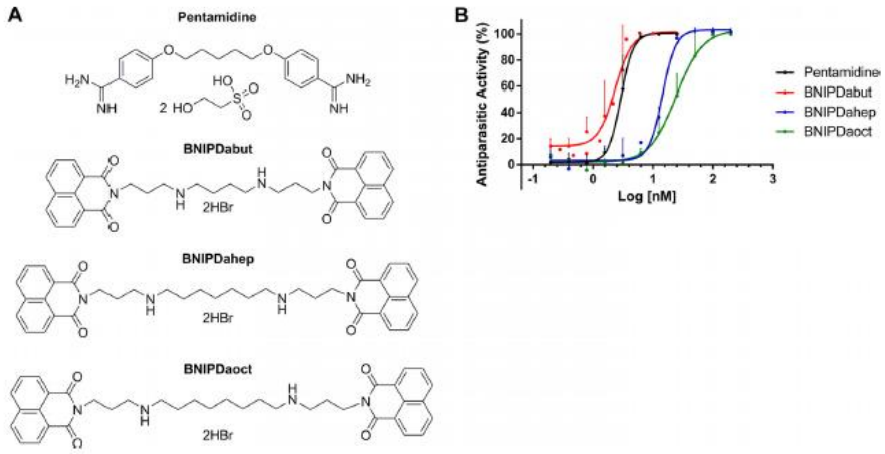

Figure 1. In vitro antitrypanosomal activity of BNIP compounds. (A) Chemical structures of pentamidine and the BNIP derivatives BNIPDabut, BNIPDahep, and BNIPDaoct. (B) Growth inhibition curves of *Trypanosoma brucei brucei* BSFs incubated in vitro with the indicated concentrations of pentamidine, BNIPDabut, BNIPDahep, or BNIPDaoct for 72 h. Parasite density was evaluated using resazurin. Dots and error bars represent the means standard deviations of antiparasitic activity. Data are from 3 independent experiments.

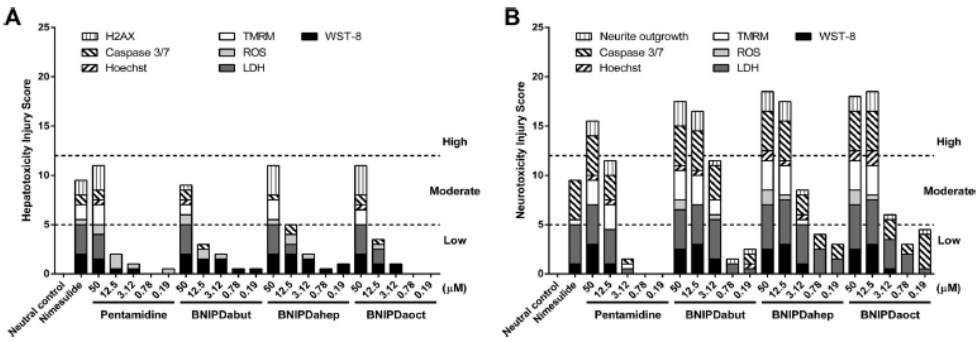

Figure 2. In vitro toxicity of BNIP derivatives. (A) Hepatotoxicity injury score. The score was calculated as the sum of individual scores obtained from a panel of in vitro cytotoxicity assays that include (i) measurement of reactive oxygen species (ROS) using CM-H2DCFDA and cell imaging by HCA, (ii) assessment of mitochondrial dysfunction measured by TMRM probe dynamics in cells and imaged by HCA, (iii) membrane integrity assayed by lactate dehydrogenase quantification, (iv) DNA damage by imaging with H2AX antibody and HCA, (v) apoptosis by caspase 3/7 activation, (vi) Hoechst staining for nuclear detection, and (vii) cell viability by WST-8 probe. Nimesulide (400 M), an approved drug with a mild toxicological profile, was included as a toxicity control. Individual scores were calculated on the basis of the relative percentage of deviation from the negative control quantified and assigned with a number from 0 to 5 (0 [0% to 20% deviation], 1 [20% to 40%], 2 [40% to 60%], 3 [60% to 100%], 4 [100% to 1,000%], or 5 [1,000% deviation]). The data represent the mean sum of these values. (B) Neurotoxicity injury score. The score was calculated in a manner similar to that for the hepatotoxicity score, but instead of DNA damage by H2AX antibody, an assay to test neurite outgrowth as imaged with an anti-tubulin III antibody and HCA was performed. The data represent the mean sum of these values. Data are from 2 independent experiments

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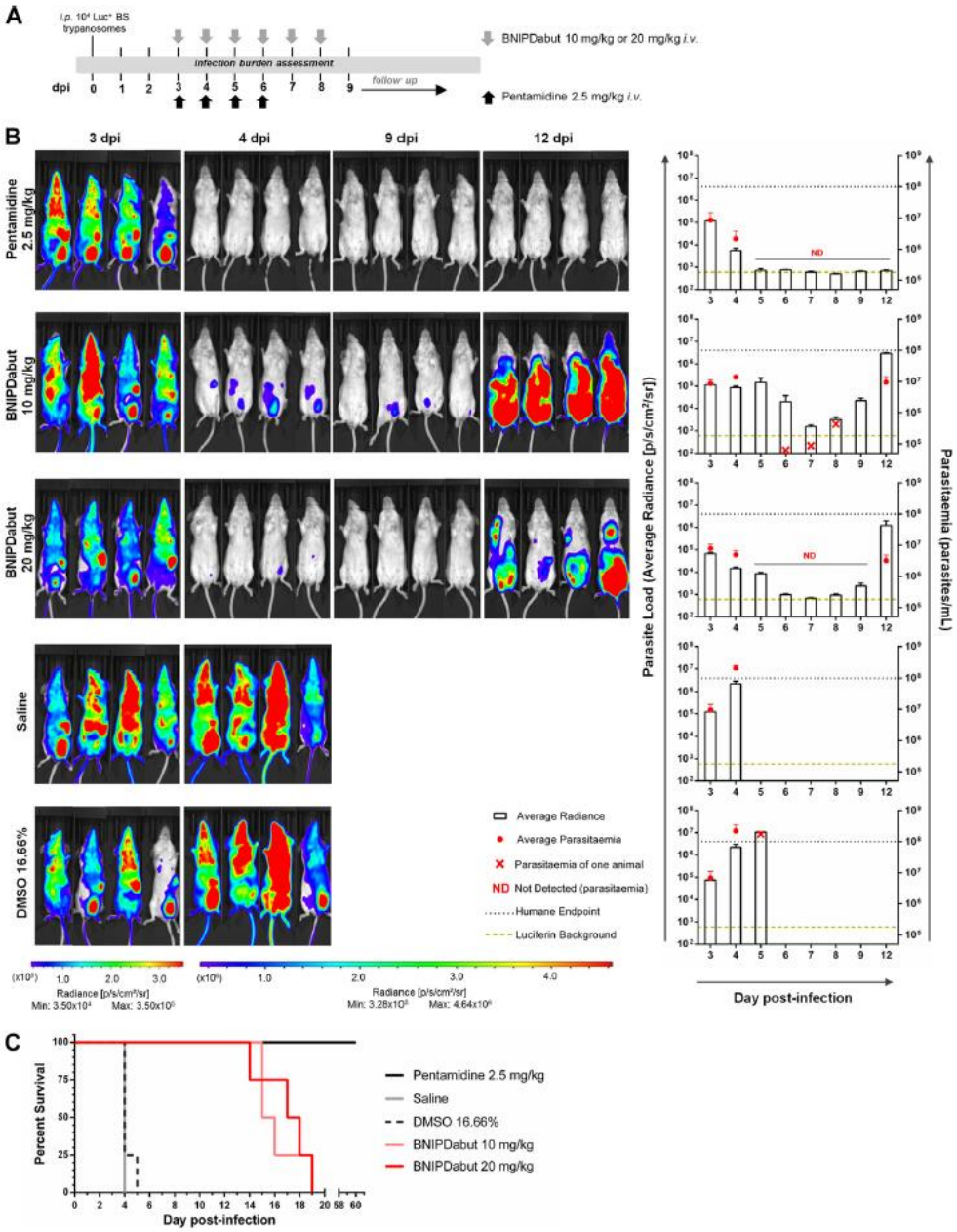

Figure 3. BNIPDabut in vivo efficacy against *T. brucei brucei*. (A) Schematic of the experimental design for evaluating the in vivo efficacy of BNIPDabut. (B) Mice were infected with 10^4 luciferase-positive BSFs by intraperitoneal injection, and the different treatments were initiated 3 days after infection. Whole-mouse bioluminescence imaging was done on days 3, 4, 9, and 12 using an IVIS Lumina LT and after injection of 2.1 mg luciferin. The bioluminescence average radiance (photons per seconds per square centimeter per steradian [p/sec/cm^2/sr]) of whole mice was quantified, and the meanstandard deviation (n=4) is shown by the bars. Parasitemia was determined with a hemocytometer, and the means + standard deviations are represented by red dots. Red X's represent the parasite concentration of the only animal in which parasites could be detected and quantified. The parasitemia detection limit was 5 x 10^4 parasites/ml. (C) Kaplan-Meyer survival curves of the infected mice treated with control and experimental doses of BNIPDabut. In panels B and C, the data are representative of 3 independent experiments

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